THE EFFECT OF TEMPERATURE ON THE RATE OF DISAPPEARANCE OF CAUDAL BANDS IN FUNDULUS HETEROCLITUS[1, 2]

FREDERICK P. FERGUSON[3]

(*Department of Zoology, University of Minnesota, and the Marine Biological Laboratory, Woods Hole*)

INTRODUCTION

It has been known since the work of Wyman (1924) that a transverse cut made near the base of the caudal fin of the killifish, *Fundulus heteroclitus* Linn., severs the nerve supply to the melanophores in the area posterior to the incision. In a white-adapted fish, this operation results in the immediate appearance of a sharply defined black band of completely dispersed melanophores, extending distally from the cut to the posterior edge of the tail.

From the experiments of Mills (1932a) and Parker (1934a) evidence has been accumulated for the hypothesis that the formation of the band is due to the mechanical stimulation, by cutting, of the melanophore-dispersing nerves to this region of the tail. Moreover, the tendency of such a band to be maintained over a period of one or more days is believed to be due to the prolonged though gradually diminishing activity of the momentarily stimulated dispersing fibers (Parker, 1934a).

If a fish with a caudal band is allowed to remain upon a white illuminated background, however, the stripe gradually blanches, the process starting at the edges and progressing inward toward the central region as an increasing number of melanophores concentrate their pigment to assume the appearance of those in normally innervated parts of the tail. This sequence of fading was first reported by Mills (1932b), who regarded it as evidence for the hypothesis that the blanching of caudal bands is due to a concentrating neurohumor secreted by the terminals of concentrating nerves in adjacent regions of the tail and capable of diffusing through the tissues into the denervated area to produce its characteristic effect upon the dispersed melanophores. This hypothesis has been actively supported by several investigators during the past decade, and is now quite generally accepted as the explanation for the blanching of caudal bands in Fundulus and in other teleosts.

The following investigation was undertaken in an attempt to determine the effect of temperature on the rate of disappearance of these caudal bands in Fundulus. A brief study of this type has been made upon the normal paradise fish, *Macropodus opercularis*, by Dalton and Goodrich (1937). These authors found that in each of four animals disappearance of the band required about one half the

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[2] Aided by grants from the Charles Peter Sigerfoos Fellowship Fund in Zoology.

[3] Present address: Department of Physiology, Louisiana State University School of Medicine, New Orleans 13, La.

time at 29° C. that it did at 20° C.[4] No other experiments of this sort have been encountered in the literature.

Material and Methods

The experiments to be reported were performed upon *Fundulus heteroclitus.* Its hardiness and availability, together with its conspicuous melanophore responses, made it an especially favorable object for the type of studies involved. Preliminary observations soon made it apparent that the more uniform size and distribution of caudal melanophores in females facilitated the accurate determination of the disappearance of caudal bands, and consequently they were employed exclusively throughout the investigation.

The fish were seined, trapped, or caught with dip nets in the brackish waters in the area of Woods Hole. Healthy females, three to four inches in length, with undamaged caudal fins were then brought to the laboratory where they were used immediately or stored temporarily in a large sink provided with running sea water. This stock of fish, as well as those used in experiments involving a period of several days, was fed every other day on the meat of the clam, *Venus mercenaria* or the edible mussel, *Mytelus edulis.*

Two constant temperature baths were employed in controlling the temperature of the water in which the fish were kept during the experiments. These baths were copper-lined tanks with capacities of approximately 5 cubic feet. Each was filled with tap water which was kept in continuous circulation to insure uniformity of temperature throughout the bath. The temperature of this water was controlled by competition between the cooling effect of a regulated stream of ice water or tap water (depending upon the temperature to be maintained) and the heating effect of a submerged resistance unit controlled by a mercury-toluene thermoregulator through a vacuum tube relay operating on A. C. With proper adjustment of the thermoregulator and the incoming stream of water, it was possible to maintain the temperature of the baths to within extreme limits of ± 0.5° C. of the desired level over the range employed in these experiments.

A wire shelf suspended 7 inches below the water level in each bath provided support for three flat-bottomed aquaria, 10 inches in diameter and 8 inches in height, which were painted white on the outside and filled with sea water to the level of the water in the bath. Each of these served as a container for four or five fish during the experiments and was aerated by a gentle stream of washed air from a small electric compressor. Illumination was provided by a 60-watt electric light bulb equipped with a white reflector and suspended 15 inches above the surface of the water of each bath. Several trial experiments were performed to determine the advisability of covering each bath with a black tent to eliminate diurnal fluctuations of light intensity. This precaution was found to be unnecessary. Moreover such an arrangement increased the difficulty of maintaining the lower temperatures, and consequently was not employed.

Caudal bands were produced according to the method of Wyman (1924), by making a transverse cut of appropriate length at the base of the tail, approximately one millimeter posterior to the last row of scales. Each animal was allowed to adapt itself completely to the white background and to the temperature at which the experiment was to be run. The head and trunk were then carefully wrapped in moist cheese cloth and the animal was laid on its right side in a large Petri dish filled with white paraffin. The protruding caudal fin was spread slightly and held flat against the paraffin by the operator's left forefinger while the cut was made. "One-ray bands" were produced by cuts one mm. in length, which severed a single fin ray and approximately half of each adjacent interray. "Two-ray bands" were produced by cuts twice this length, and included two rays, the interray between them, and approximately half of each interray bounding their outer sides. As already noted by many investigators, the large majority of these operations are unaccompanied by any disturbance of blood supply to the denervated area except for the region immediately surrounding the cut.

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[4] When calculated from their data, the mean Q_{10} for the group is found to be 2.27.

After the operation each fish was carefully measured from the tip of its snount to the conspicuous arc of blood vessels (Fries, 1931) at the base of the tail. The length to the nearest millimeter was then recorded, together with a sketch indicating the shape and other characteristics of the tail and the position of the band. Such a procedure proved very valuable for insuring future identification of the individual.

After this treatment the fish was returned to its white aquarium in the constant temperature bath, where the band was allowed to fade. The early progress of fading was followed by macroscopic observations at various intervals. Unhealthy animals as well as those whose tails became injured in any way to affect the band were always discarded as soon as they were noticed. When the band became rather faint, subsequent observations were made in so far as possible at intervals of two hours. More frequent observations were generally impractical because of the injurious effects of excessive handling and inability to perceive definite changes in the state of most bands over intervals of less than two hours.

Final stages of blanching were always observed with the aid of a binocular dissecting microscope (10X oculars, 32 or 48 mm. objectives), a method which was found to be more reliable than the use of the unaided eye. Since the blanching is a gradual process, it is of course impossible to determine the exact end point by observations made at intervals of time. Consequently, the mid-point between the time of the last observation in which pigment dispersion was manifested by any of the band melanophores and the first one in which all of these cells had concentrated their pigment as completely as those in the normally innervated regions of the tail was taken as the best estimate of the time of complete fading. Thus, if a band were still very slightly evident 24 hours after its formation, but had completely disappeared by the time of the next observation two hours later, the blanching time would be recorded as 25 hours. Confirmatory observations were then continued for several hours.

RESULTS

Preliminary. Before proceeding with experiments on the relation of temperature to the rate of disappearance of caudal bands, studies were made to determine whether or not an individual Fundulus manifests a more or less characteristic reaction time at a particular temperature level. The advisability of such a study was emphasized by the fact that a great deal of variation had been found to occur among different individuals under the same environmental conditions. Thus, for instance, in a group of 52 Fundulus with one-ray bands the blanching time at 20° C. varied from 16 to 197 hours; in a group of 17 at 25°, it varied from 13 to 138 hours; and in a third group of 48 at 30°, it varied from 4 to 66 hours.

In determining the degree of constancy of blanching time for an individual, a single one-ray band was produced and its time of disappearance recorded. The fish was then allowed to remain in its white aquarium, and after a few hours a second band was made by cutting another ray a few millimeters above or below the faded area. The time required for disappearance of this second band was then determined without reference to that required by the first one.

It was found that, with few exceptions, the time required for the disappearance of the two bands was remarkably similar. In the majority of cases the difference lay between zero and 2 hours. For a group of 25 fish at 30° C., the mean difference in time of disappearance of the first and second bands was 0.54 ± 1.07[5] hours. From these data the significance of the mean difference can be estimated from the probability value (P) corresponding to the familiar "t" criterion.[6] For this experiment $t = 0.505$, corresponding to a P value of something over 0.55. Thus, the chances of such a difference being exceeded through errors of random sampling are greater than 55 per cent, and consequently it cannot be regarded as significant. Similar results were obtained with smaller numbers of fish at 15°, 20°, and 25° C. and it was therefore concluded that at a given temperature level there is no significant difference in the blanching time of two one-ray caudal bands produced at different times upon the same fish.

It might be of interest to note that one set of determinations was carried out in such a way as to compare the time of blanching of one-ray bands in sea water and in fresh water at 30° C. During the fading of the first band the fish were kept in sea water, but this was replaced by ordinary tap water for the second run. Of eight individuals successfully carried through the experiment, the mean difference in blanching time of the two bands was 0.44 ± 0.63 hours. The probability of such a difference being exceeded through errors of random sampling was found to be 0.5076 (50.76 per cent) and it is therefore apparent that the rate of disappearance of bands in fresh water is not significantly different from that in sea water.

Temperature. With these preliminary studies in mind, the problem of determining the effect of temperature on the rate of the fading reaction was undertaken. In carrying out these experiments, the time required for blanching at one temperature was determined as usual, and the fish was then transferred to the second bath, the temperature of which was regulated at 10° C. higher or lower than that of the first. The animal was allowed to adapt itself gradually to the new temperature. After a few hours a new band was produced above or below the site of the original and the time of its disappearance determined. In view of the preliminary work described above, any significant difference in the time required for disappearance of the two bands could be reasonably ascribed to the change in temperature.

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[5] Standard error of mean. In this paper, standard deviation has been calculated from the formula $s_x = \sqrt{\frac{\Sigma x^2}{N} - \bar{x}^2}$ (Treloar, 1939, p. 55), in which s_x represents the standard deviation; Σx^2 the sum of the squares of each variate; $\bar{x}^2$ the square of the mean; and N the number of individuals in the series. The standard error of the mean has been calculated as $\text{S.E.}_{\bar{x}} = \frac{s_x}{\sqrt{N - 1}}$ (Treloar, 1939, p. 138).

[6] Calculated from the equation $t = \frac{\bar{d}}{\text{S.E.}_{\bar{d}}}$ (Treloar, 1942, p. 54), in which $\bar{d}$ represents the mean difference between the pairs of values, and $\text{S.E.}_{\bar{d}}$ the standard error of that mean difference. Corresponding values of P were obtained from Treloar's (1936) extensive table of probability in terms of t.

Table I shows the results obtained in 17 successful cases in which the first band was allowed to fade at 15° and the second at 25° C. In the column under the heading "Fish" the animals have been arranged arbitrarily in order of increasing values of Q_{10}, recorded in the last column. It can be seen that in every case the blanching reaction required much less time at the higher temperature, the difference being designated in the appropriate column. From 133.76 ± 15.65 hours at 15° the mean time required decreased to 46.94 ± 6.08 hours at 25° C. The value of t calculated from the mean difference is beyond the tabulated range, indicating a probability of less than 1 : 10,000 that such a difference would be exceeded through errors of random sampling.

TABLE I

Time in hours required for blanching of one-ray caudal bands at 15° and at 25° C. on the same fish. First run at 15°, second at 25°

Range, interval during which final blanching occurred; Mp, mid-point of that interval; S.D., standard deviation; S.E., standard error of mean.

Fish	Run 1: 15°		Run 2: 25°		Difference M_{p15}–M_{p25} (hrs.)	Q_{10}
	Range (hrs.)	M_{p15} (hrs.)	Range (hrs.)	M_{p25} (hrs.)		
1	88–90	89	62–66	64	25	1.39
2	74–76	75	42–44	43	32	1.74
3	44–46	45	22–24	23	22	1.96
4	114–116	115	56–58	57	58	2.02
5	44–46	45	20–22	21	24	2.14
6	268–276	272	124–126	125	147	2.18
7	98–100	99	38–48	43	56	2.30
8	98–100	99	38–48	43	56	2.30
9	190–192	191	66–68	67	124	2.85
10	172–182	177	54–56	55	122	3.22
11	170–172	171	52–54	53	118	3.23
12	76–78	77	18–24	21	56	3.67
13	184–186	185	46–48	47	138	3.94
14	88–90	89	20–22	21	68	4.24
15	182–186	184	40–42	41	143	4.49
16	212–216	214	46–48	47	167	4.55
17	146–148	147	26–28	27	120	5.44
Mean	133.76		46.94		*86.82	3.04
S.E.	±15.65 hrs.		±6.08 hrs.		±12.06 hrs.	±0.285
S.D.	62.61 hrs.		24.32 hrs.		48.23 hrs.	1.14

* $t = 7.20$; $P = <.0001$.

More informative than the actual difference in time required at the two temperature levels, however, are the Q_{10} values given in the last column of the table. These were found to vary about a mean of 3.04 ± 0.285, indicating that on the average the rate of blanching of caudal bands was approximately trebled by increasing the temperature from 15° to 25° C.

The results obtained upon 14 fish in which the first one-ray band was allowed to fade at 20° and the second at 30° C. are shown in Table II. Here again the rise of 10° produced a marked increase in the rate of the reaction in every case, the mean Q_{10} value being 2.18 ± 0.166.

TABLE II

Time in hours required for blanching of one-ray caudal bands at 20° and at 30° C. on the same fish. First run at 20°, second at 30°

Range, interval during which final blanching occurred; Mp, mid-point of that interval; S.D., standard deviation; S.E., standard error of mean.

Fish	Run 1: 20°		Run 2: 30°		Difference $Mp_{20}-Mp_{30}$ (hrs.)	Q_{10}
	Range (hrs.)	Mp_{20} (hrs.)	Range (hrs.)	Mp_{30} (hrs.)		
1	35–36	35.5	22–24	23	12.5	1.54
2	36–44	40	24–26	25	15	1.60
3	39–41	40	21–23	22	18	1.82
4	37–39	38	19–21	20	18	1.90
5	84–86	85	38–48	43	42	1.98
6	36–40	38	18–20	19	19	2.00
7	43–45	44	21–23	22	22	2.00
8	51–55	53	25–27	26	27	2.04
9	22–24	23	10–12	11	12	2.09
10	72–74	73	34–36	35	38	2.09
11	78–80	79	36–38	37	42	2.14
12	46–48	47	20–22	21	26	2.24
13	84–93	88.5	26–28	27	61.5	3.28
14	56–58	57	14–16	15	42	3.80
Mean[7]	52.93		24.71		*28.21	2.18
S.E.	±5.48 hrs.		±2.30 hrs.		±3.93 hrs.	±0.166
S.D.	19.79 hrs.		8.32 hrs.		14.17 hrs.	0.60

[7] Discrepancy of 0.01 between difference of means and mean difference due to rounding off figures at second decimal place.

* $t = 7.18$; $P = <.0001$.

A similar relation of temperature to the rate of fading was found in a series of seven Fundulus in which one two-ray band was allowed to disappear at 20° and the second at 30° C. The mean time required at the lower temperature was 126.79 ± 11.31 hours, whereas that at the higher level was 63.29 ± 8.24 hours, the mean difference being 63.50 ± 6.14 hours. It will be noted that the blanching time of these bands at each temperature was much longer than that of the one-ray bands at the same level (cf. Parker, 1934b). The Q_{10} values varied about a mean of 2.09 ± 0.16, however, indicating that in these bands, as in the narrower ones, fading occurs approximately twice as rapidly when the temperature is raised from 20° to 30° C.

Table III shows the results of another experiment which was also carried out at 20° and at 30° C., but with the temperature sequence reversed. In this case the first determination of blanching time was made at 30° and the fish were then transferred to the lower temperature. The results were in all respects similar to those of the preceding experiments, a much longer time being required by each fish for the disappearance of the band at the lower temperature. From a consideration of the Q_{10} values it can be seen that in the majority of cases the rate of the reaction was more than twice as high at the 30-degree level as at 20°, the mean ratio being 2.59 ± 0.14.

TABLE III

Time in hours required for blanching of one-ray caudal bands at 20° and at 30° C. on the same fish. First run at 30°, second at 20°

Range, interval during which final blanching occurred; Mp, mid-point of that interval; S.D., standard deviation; S.E., standard error of mean.

Fish	Run 1: 30°		Run 2: 20°		Difference Mp_{20}–Mp_{30} (hrs.)	Q_{10}
	Range (hrs.)	Mp_{30} (hrs.)	Range (hrs.)	Mp_{20} (hrs.)		
1	15–17	16	24–26	25	9	1.56
2	23–24	23.5	36–48	42	18.5	1.79
3	24–26	25	46–50	48	23	1.92
4	31–33	32	62–64	63	31	1.97
5	23–24	23.5	46–48	47	23.5	2.00
6	23–25	24	47–49	48	24	2.00
7	23–24	23.5	47–49	48	24.5	2.04
8	51–53	52	108–120	114	62	2.19
9	18–20	19	41–43	42	23	2.21
10	17–19	18	40–42	41	23	2.28
11	27–31	29	65–72	68.5	39.5	2.36
12	23–24	23.5	55–57	56	32.5	2.38
13	22–24	23	52–62	57	34	2.48
14	14–23	18.5	48–50	49	30.5	2.65
15	15–17	16	42–44	43	27	2.69
16	25–27	26	70–72	71	45	2.73
17	25–27	26	73–75	74	48	2.85
18	15–17	16	46–48	47	31	2.94
19	13–15	14	43–45	44	30	3.14
20	25–29	27	87–91	89	62	3.30
21	25–29	27	98–109	103.5	76.5	3.83
22	11–13	12	46–50	48	36	4.00
23	13–14	13.5	55–57	56	42.5	4.15
Mean	22.96		57.57		*34.61	2.59
S.E.	±1.73 hrs.		±4.39 hrs.		±3.31 hrs.	±0.14
S.D.	8.10 hrs.		20.59 hrs.		15.34 hrs.	0.67

* $t = 10.46$; $P = <.0001$.

Discussion

In *Fundulus heteroclitus*, the time required for the complete blanching of caudal bands of equal width varies greatly among different individuals, even when maintained under identical environmental conditions during the process. This variation was apparent in the preliminary studies of this investigation and was confirmed by all subsequent work. On the other hand, each fish manifests a characteristic reaction time for the process. Thus, two bands of equal width produced upon the same individual were found to fade at remarkably similar rates under the same environmental conditions, the difference in time required for their disappearance being of no statistical significance.

This characteristic reaction time for individuals makes it possible, by performing "paired" experiments upon each of a number of animals, to determine with considerable accuracy the relation of a particular variable factor to the time required for the fading of caudal bands in Fundulus. In this way one band of each animal serves in effect as a control for the other and any significant difference between the blanching time of the two can be reasonably attributed to the factor which has been varied. This method was employed in the present investigation to determine the effect of temperature upon the rate of the fading reaction.

The rate of fading of caudal bands in Fundulus was found to be influenced directly by temperature, a rise of 10° over the intervals 15 — 25° C. or 20 — 30° C. resulting in an average increase of 2 to 3 times in the speed of the reaction. The results obtained in this investigation thus agree in this respect with those of Dalton and Goodrich (1937) on the paradise fish, *Macropodus opercularis*. These authors ascribed their results to the effects of temperature upon the rate of diffusion of the concentrating neurohumor into the band from adjacent regions of the tail. This interpretation would admittedly add considerable significance to the immediate value of results of this sort in the light of the neurohumoral hypothesis. Unfortunately, however, such a conclusion is difficult to reconcile with the prevalent concept that the effect of temperature changes upon the rate of diffusion processes is ordinarily of considerably less magnitude than the effect observed upon the rate of disappearance of caudal bands in their study and in the present investigation. Jacobs (1928), for instance, states that ". . . processes whose rate is limited by the diffusion of dissolved substances (give) values of Q_{10} in the vicinity of 1.25 or 1.3; and chemical reactions of the ordinary sort values which are usually in excess of 2."

Hence, there is no *a priori* reason to regard the blanching reaction as one limited by diffusion, since its temperature coefficient falls within the range generally attributed to chemical reactions. This is not meant to imply, however, that diffusion is to be excluded from the total reaction. The blanching of caudal bands is undoubtedly a complex phenomenon, involving many different processes, several or possibly all of which may be affected by temperature. Consequently, the observed results may well represent the net effect of temperature upon the rate of the reaction as a whole rather than its influence upon any single process involved. It therefore seems advisable to regard the temperature coefficient of this reaction, like that of many other complex biological reactions, as being primarily of descriptive rather than of analytical value.

SUMMARY

1. In *Fundulus heteroclitus*, the time required for the blanching of caudal bands of equal width varies greatly among different individuals under the same environmental conditions.

2. Each fish manifests a characteristic reaction time, however, and it is therefore possible, by performing "paired" experiments, to determine the effect of certain variable factors upon the rate of the blanching reaction in any individual.

3. By this method it has been found that the rate of blanching of caudal bands in Fundulus is directly influenced by temperature. A rise of 10° over the interval of 15° to 25° C. increased the speed of the reaction by an average of 3.04 ± 0.285 times in a group of 17 fish with one-ray bands. A similar rise over the interval of 20° to 30° C. increased it by an average of 2.18 ± 0.166 times in a group of 14 animals with one-ray bands, and 2.09 ± 0.16 times in seven animals with two-ray bands. Conversely, a decrease from 30° to 20° C. decreased the rate by an average of 2.59 ± 0.14 times in a group of 23 fish with one-ray bands.

It is suggested that these results be ascribed to the net effect of temperature upon the rate of the blanching reaction as a whole rather than to its influence upon any single process involved.

4. By the same method it was also found that the rate of blanching of caudal bands in fresh water is the same as that required in sea water. For a group of 8 animals with one-ray bands, the mean difference in reaction time in these two media at 30° C. was 0.44 ± 0.63 hours.

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